

LAW OFFICES

LYON & LYON LLP

A LIMITED LIABILITY PARTNERSHIP
INCLUDING PROFESSIONAL CORPORATIONS

SUITE 660

4250 EXECUTIVE SQUARE

A JOLLA, CALIFORNIA 92037

FAX (619) 552-0159

TELEPHONE (619) 552-8400

FREDERICK S LYON (1873-1948)

LEONARD S LYON (1896-1964)

LOS ANGELES, CA OFFICE

633 WEST FIFTH STREET, SUITE 4700
LOS ANGELES, CALIFORNIA 90071-2066
FAX (213) 955-0440
TELEPHONE (213) 489-1600

COSTA MESA, CA OFFICE

3200 PARK CENTER DRIVE, SUITE 1200
COSTA MESA, CALIFORNIA 92626
FAX: (714) 751-8209
TELEPHONE (714) 751-6606

SAN JOSE, CA OFFICE

303 ALMADEN BOULEVARD, SUITE 1150
SAN JOSE, CALIFORNIA 95110
FAX: (408) 287-2664
TELEPHONE (408) 993-1555

OF COUNSEL

BRADFORD J DUFT
SUZANNE L BIGGS
STEPHEN S KORNICZKY
DANIEL N YANNUZZI**

* A PROFESSIONAL CORPORATION
** MEMBER OF DISTRICT OF COLUMBIA BAR
*** MEMBER OF NEW JERSEY BAR

May 26, 1998

Attorney Docket No. 234/293

BOX Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Enclosed is a continuation patent application as
follows:

Applicants: WILLIAM JOHN MARTIN

Title: STEALTH VIRUS DETECTION IN THE CHRONIC FATIGUE
SYNDROME

CERTIFICATE OF MAILING

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as Express Mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

EM 104358581 US

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Assistant Commissioner for Patents
May 26, 1998
Page 2

Attorney Docket No. 203/128

No. Pages of Specification: 20

No. Pages of Claims: 4

No. Pages of Abstract: 1;

No. Pages of Sequence Listing 0;

No. Pages of Drawings: 0; and

No. Sheets of Tables: 0.

If this application is found otherwise to be incomplete, or if at any time appears that a telephone conference with counsel would helpfully advance prosecution, please telephone the undersigned in La Jolla, California (619) 552-8400.

Please direct all correspondence to the following:

Richard J. Warburg, Esq.
LYON & LYON LLP
633 West Fifth Street
Suite 4700
Los Angeles, CA 90071

Kindly acknowledge receipt of the foregoing application by returning the self-addressed postcard.

Respectfully submitted,

LYON & LYON LLP



Charles S. Berkman
Registration No. 38,077

CSB:ca
633 West Fifth Street
Suite 4700
Los Angeles, CA 90071
(619) 552-8400
Enclosures

**UTILITY
PATENT APPLICATION
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No

234/293

First Named Inventor or Application Identifier Martin, William J.

Title

STEALTH VIRUS DETECTION IN THE CHRONIC FATIGUE SYNDROME

Express Mail Label No.

EM104358581US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

ADDRESS TO:

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1. ☒ * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages 25]
(preferred arrangement set forth below)
- Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☐ Drawing(s) (35 USC 113) [Total Sheets ☐
4. Oath or Declaration [Total Pages ☐

a. ☐ Newly executed (original or copy)

b. ☒ Copy from a prior application (37 CFR § 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]

i. ☐ **DELETION OF INVENTOR(S)**
Signed statement attached deleting
inventor(s) named in the prior application,
see 37 CFR §§ 1.63(d)(2) and 1.33(b).

5. ☒ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application, from which a
copy of the oath or declaration is supplied under Box 4b, is
considered as being a part of the disclosure of the
accompanying application and is hereby incorporated by
reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
- a. ☐ Computer Readable Copy
- b. ☐ Paper Copy (identical to computer copy)
- c. ☐ Statement verifying identity of above copies.

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR § 3.73(b) Statement ☒ Power of Attorney
(when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure
Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
14. ☐ * Small Entity
Statement(s) ☒ Statement filed in prior application,
(PTO/SB/09-12) Status still proper and desired
15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
16. ☒ Other: Check for \$ 573.00.

*** NOTE FOR ITEMS 1&14: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY
FEES, A SMALL ENTITY STATEMENT IS REQUIRED FOR (37 C.F.R. § 1.27),
EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON
(37 C.F.R. § 1.28).**

17. If a **CONTINUING APPLICATION**, check appropriate box, and supply the requisite information below and in a preliminary amendment:


☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: 08 /157,811
Prior application information: Examiner CARTER, P Group/Art Unit 1802

18. CORRESPONDENCE ADDRESS

Customer Number of Bar Code Label

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or ☒ Correspondence address below

NAME	Charles S. Berkman, Esq				
	LYON & LYON				
ADDRESS	633 West Fifth Street, Suite 4700				
CITY	Los Angeles	STATE	CA	ZIP CODE	90071
COUNTRY	US	TELEPHONE	619/552-8400	FAX	213/955-0440
Name (Print/Type)	Charles S Berkman		Registration No. (Attorney/Agent)	38,077	
Signature			Date	May 26, 1998	

FEE TRANSMITTAL

Note: Effective October 1, 1997.
Patent fees are subject to annual revision.

Complete if Known

Application Number	To be assigned
Filing Date	May 26, 1998
First Named Inventor	William John Martin
Group Art Unit	To be assigned
Examiner Name	To be assigned
Attorney Docket Number	234/293

Total Amount of Payment **(\$ 573.00)**

METHOD OF PAYMENT (check one)

1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:
Deposit Account Number 12-2475
Deposit Account Name Lyon & Lyon LLP
☒ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17 ☐ Charge the Issue Fee Set in 37 CFR 1.18 at the Mailing of the Notice of Allowance
2. ☒ Payment enclosed: \$573.00
☒ Check ☐ Money Order ☐ Other

FEE CALCULATION

1. FILING FEE

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Paid
101	790	201	395	Utility filing fee	\$395.00
106	330	206	165	Design filing fee	
107	540	207	270	Plant filing fee	
108	790	208	395	Reissue filing fee	
114	150	214	75	Provisional filing fee	
SUBTOTAL (1) (\$)					\$395.00

2. CLAIMS

	Extra	Fee from below	Fee Paid
Total Claims	25	-20 = 5	X 11 = 55.00
Independent Claims	6	-3 = 3	X 41 = 123.00
Multiple Dependent Claims		X	=

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description
103	22	203	11	Claims in excess of 20
102	82	202	41	Independent claims in excess of 3
104	270	204	135	Multiple dependent claim
109	82	209	41	Reissue independent claims over original patent
110	22	210	11	Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$) 178.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action.	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action.	
115	110	215	55	Extension for reply within first month.	
116	400	216	200	Extension for reply within second month	
117	950	217	475	Extension for reply within third month.	
118	1,510	138	1,510	Extension for reply within fourth month.	
128	2,060	228	1,030	Extension for reply within fifth month.	
119	310	219	155	Notice of Appeal	
120	310	220	155	Filing a brief in support of an appeal	
121	270	221	135	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,320	241	660	Petition to revive - unintentional	
142	1,320	242	660	Utility issue fee (or reissue)	
143	450	243	225	Design issue fee	
144	670	244	335	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	790	246	395	Filing a submission after final rejection (37 CFR 1.129(a))	
149	790	249	395	For each additional invention to be examined (37 CFR 1.129(b))	

Other fee (specify) _____

Other fee (specify) _____

* Reduced by Basic Filing Fee Paid

SUBTOTAL (3) \$ -0-

SUBMITTED BY

Typed or Printed Name Charles S. Berkman

Signature [Signature]

Date May 26, 1998

Complete (if applicable)

Reg. Number 38,077

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DESCRIPTION

STEALTH VIRUS DETECTION IN THE CHRONIC FATIGUE SYNDROME

This application is a continuation-in-part of co-
pending United States patent application Serial No.
07/887,502, filed May 22, 1992 entitled "Stealth Virus
Detection in the Chronic Fatigue Syndrome" which is a
5 continuation-in-part application of United States patent
applications Serial No. 07/704,814, filed May 23, 1991;
and Serial No. 07/763,039, filed September 20, 1991
entitled "Spumavirus detection in the chronic fatigue
syndrome". These prior submissions, including any
10 drawings, are incorporated by reference in their entirety,
herein.

1. Field of Invention

The present invention relates generally to methods
for detecting the presence of a virus. More particularly,
15 it relates to the detection of viruses by tissue culture
techniques.

2. Background of the Invention

A. Chronic Fatigue Syndrome

Palca, *Science*, 249:1240-1241 (1990) and Palca,
20 *Science*, 254:1726-1728 (1991) describe attempts to
identify a causative agent for chronic fatigue syndrome.

DeFreitas et al., *Chemical Abstracts*, 114: Abstract
No. 205331c (1991) describes retroviral sequences related
to human T lymphotropic virus type 2 in patients with
25 chronic fatigue immune dysfunction syndrome.

Gupta et al., *Scandinavian Journal of Immunology*,
33:319-327 (1991) describes a comprehensive immunological
analysis of chronic fatigue syndrome. The analysis of
cell mediated and antibody mediated immunity was performed
30 in 20 patients with chronic fatigue syndrome and 20 age
and sex matched healthy controls.

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B. Culture Techniques

Werner, *Lancet*, II:258-259 (1979) describes the isolation of foamy viruses from patients with de Quervain Thyroiditis and the detection of a cytopathic effect.

- 5 Freshney, *Culture of Animal Cells: A Manual of Basic Technique*, pp. 127-136 (Alan R. Liss Inc., 1987) describes the maintenance of cell cultures and states that four factors indicate the need for replacement of culture medium: (1) a drop in pH; (2) high cell concentration;
10 (3) cell types such as transformed cells, continuous cell lines and some embryonic cells that deteriorate rapidly at high cell densities; and (4) morphological deterioration of the cell such as granularity around the nucleus, cytoplasmic vacuolation, and rounding up of the cells with
15 detachment from the substrate.

DiLuca et al., *Virology*, 175:199-210 (1990) describes the replication of viral and cellular DNA in human herpesvirus 6-infected cells and the use of medium RPMI 1640 containing 10% fetal calf serum.

- 20 Ablashi et al., *International Journal of Cancer*, 42:787-791 (1988) describes the use of human hematopoietic cell lines for the propagation of HBLV (human herpesvirus 6) in RPMI 1640 supplemented with 10% FBS and antibiotics.

- Rethwilm et al., *Nucleic Acids Research*, 18:733-738
25 (1990) describes an infectious molecular clone (pHSRV) of the human spumaretrovirus (HSRV). pHSRV derived virus produced foamy virus typical cytopathic effects in susceptible cultures.

3. Summary of the Invention

The present invention provides a method for detecting a stealth virus by culturing a sample under conditions that induce a cytopathic effect. Several culture
5 conditions are sufficient to induce a cytopathic effect in a sample containing a stealth virus. These culture conditions include replacing the culture medium every 24 to 72 hours, adding 5% to 10% fetal calf serum to the culture medium, using serum free medium X Vivo-15, using
10 preculture centrifugation and adding viral enhancing medium to the culture.

In one aspect, a method of detecting a stealth virus is provided by culturing a sample under conditions in which any stealth virus in the sample is able to induce a
15 cytopathic effect.

The term "stealth virus" refers to a virus having all of the following characteristics: (a) the ability to induce a cytopathic effect in fibroblastic cultures including primary kidney cell cultures which is
20 characterized by the production of foamy appearing cells, including cell syncytia; (b) the ability to produce a toxin capable of suppressing viral growth; (c) the ability to grow in cells from a plurality of species; (d) the inability of viral infected cells to react in a typical
25 manner using typing antisera specific for cytomegalovirus, herpes simplex virus, varicella zoster virus, Epstein-Barr virus and human T cell lymphotropic virus (HTLV); (e) the inability of viral infected cells to hybridize in a typical manner with nucleic acids probes specific for
30 HTLV, cytomegalovirus, herpes simplex virus, human herpes virus-6, varicella zoster virus and Epstein-Barr viruses using stringent hybridization conditions; and (f) the inability to evoke an inflammatory response in tissues which it infects.

35 The term "cytopathic effect" (CPE) refers to the appearance of rounded, slightly enlarged, refractile cells

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throughout the culture. In some cultures the CPE progresses to very prominent collections of tightly packed, enlarged, foamy-cell appearing cells, with clearly defined cell syncytia and evidence of considerable cell destruction. Stages between the spindle shape of the normal fibroblasts and the rounded appearance of affected cells can be seen. Several inclusions, consistent with vacuoles, can be seen within the cytoplasm. As their numbers increase, affected cells form several tightly adherent clumps with indistinct cell boundaries. The affected cells continue to proliferate and scatter away from the cell clumps.

In preferred embodiments the presence of the stealth virus is detected by (1) inoculating a permissive cell line with a sample, and (2) detecting *in vitro* a CPE in the permissive cell line; the cell line is maintained in culture medium that is replaced every 24 to 72 hours; the cell line is an insect cell line e.g., the cell line is derived from a plurality of species and may even include *Spodoptera fruiperdo* derived ovarian cell line Sf9; and the sample is from a patient suspected of having chronic fatigue syndrome (CFS) based upon other recognized criteria well known to physicians in the art.

The term "chronic fatigue syndrome" (CFS) refers to an illness whose major characteristic is an unexplained fatigue lasting beyond 6 months which results in greater than 50% reduction in an individual's normal level of activity (Holmes et al., "Chronic fatigue syndrome: A working case definition," *Ann. Intern. Med.*, 108:387-389 (1988); Holmes, "Defining the chronic fatigue syndrome", *Rev. Inf. Dis.*, 13 (Suppl. I):S53-5 (1991); Shafan, "The chronic fatigue syndrome" *Am. J. Med.*, 90:731-738 (1991)). To establish a clinical diagnosis, the patients should show evidence of suffering at least eight of the following minor symptoms: fever, sore throat, myalgia, muscle weakness (which may be exacerbated by exercise),

arthralgia, lymphadenopathy, sleep disturbance, headaches, acute or subacute onset, and neuropsychological symptoms. The neuropsychological symptoms include a difficulty in thinking, dysnomia, confusion, forgetfulness, irritability, depression, photophobia and transient visual scotomata.

A cell is "permissive" if a particular virus causes a productive infection in it. A productive infection is a viral infection of a cell that produces progeny with the vegetative or lytic cycle. A productive infection by a stealth virus is characterized by the appearance of a cytopathic effect. Using appropriate conditions, stealth viruses infect and propagate in many mammalian cells *in vitro*, especially cultures of fibroblastic cells including primary kidney cell cultures, and other cell lines of epidermal, mesodermal, neuroectodermal and lymphoid origin, e.g., glial cells, myoblasts, etc. The distinctive CPE is, however, readily observed in primary fibroblast cultures. At present, therefore, these are the preferred cell lines to isolate the virus from clinical and animal samples. Suitable cells include human MRC-5 lung fibroblasts, primary human foreskin fibroblasts MRHF and rhesus monkey kidney RMK cells. These well known cell lines are available commercially (e.g., from Whittaker Bioproducts, Walkersville, Maryland). It should be noted that not all cell lines are permissive for growth and/or for the expression of a cytopathic effect (CPE). Conversely, other cell lines may be more efficient in promoting the growth of stealth viruses than the fibroblast cell lines currently used. The tissue culture cell lines are maintained in the laboratory using methods well known in the art.

In a second aspect a method of detecting a stealth virus is provided by: (1) inoculating a permissive cell line with a sample; (2) neutralizing stealth virus-

associated toxic activity; and (3) detecting *in vitro* a cytopathic effect in the permissive cell line.

The term "stealth virus-associated toxin" or "toxin" refers to the molecular entity (or entities) that mediates the toxic activity observed in stealth virus cultures *in vitro*, and which appears capable of suppressing stealth virus growth. Cultures with toxin are often more difficult to infect with other stealth viral isolates. The toxic activity is detectable in serum and cerebrospinal fluid from stealth virus infected patients and may mediate certain symptoms associated with the patient's illness.

In preferred embodiments the toxic activity is neutralized by frequently replacing the culture medium, using medium in which the production of the toxin is minimized, or by adding a neutralizing agent to the culture; the neutralizing agent is fetal calf serum or retinoic acid, an antibody specific for the toxin, or a chemical or virus derived agent capable of competing with or reversing the toxic activity; and the sample is derived from a human, animal, food, or other environmental substance or object.

The term "neutralized" refers to any amount of inhibition or decrease. Thus, by neutralizing the toxic activity, one actually increases the detection of stealth virus. The term "replacing" refers to removing old culture medium and transferring new culture medium into the sample being tested to a new culture medium.

Stealth viruses may be isolated from a sample of blood or other biological samples including surgical and fine needle aspiration tissue biopsies, post mortem organ biopsies, throat swabs and saliva, urine, cerebrospinal fluid (CSF), other body fluids, blood and blood products intended for transfusion or for *in vitro* uses, vaccines, foods, and from the environment.

In a third aspect, a method for detecting a stealth-virus is provided by: (1) inoculating a permissive cell line with a sample of the virus in a culture; (2) adding serum free medium to the culture; and (3) detecting in
5 *vitro* the presence of a CPE in the permissive cell line.

In preferred embodiments, the serum free medium is medium X Vivo-15 (BioWhittaker, Inc., Walkersville, Maryland). X Vivo-15 is a Iscove's modified Delbecco's medium with the addition of human albumin, human insulin
10 and human transferrin. Iscove's modification of Delbecco's medium is intended to support high density cell growth and has additional glucose, salts and vitamins compared to Delbecco's medium (Iscove and Melchers, J., *Experimental Medicine*, 147:923 (1963)). Albumin, insulin
15 and transferrin are provided as a replacement for the need for serum. Since these proteins are of human origin, X Vivo-15 is suitable for growing human cells intended to be injected into autologous recipients.

Of several serum free media tested, medium X Vivo-15
20 was clearly superior to medium 199 plus 7% FCS for supporting stealth viral growth. The CPE developed more rapidly and there was less of a general loss of vitality seen with the usual stealth viral cultures. Medium X Vivo-15 was also found to be superior to other serum free
25 media, for example, Aims-V medium from GIBCO BRL, Gaithersburg, Maryland. Aims-V medium also uses human albumin, insulin and transferrin, but the basic salt component is F-10 medium (Ham, *Exp. Cell Res.*, 29:515 (1963)), rather than Iscove's. X Vivo-15 was also
30 superior to medium 199 plus bovine albumin, insulin and transferrin, with or without FCS.

In a fourth aspect a method of detecting a stealth virus is provided by: (1) co-centrifuging a sample of said virus with a permissive cell line of indicator cells;
35 (2) inoculating the cell mixture into culture vessels; and (3) detecting *in vitro* a CPE in the permissive cell line.

Pre-culture centrifugation (PCC) of patients' lymphoid cells with indicator fibroblast cells refers to co-centrifugation of patients' or animals' lymphocytes with freshly harvested fibroblasts and replating the cells
5 back into the original tubes containing the fibroblasts.

In preferred embodiments the method involves adding cytomegalovirus (CMV) supernatant, or viral enhancing medium, to the culture and frequently refeeding the culture medium. Viral enhancing medium (VEM) is derived
10 from the supernatants from actively replicating viruses which are functionally related to the stealth virus. Filtered, boiled medium collected from a CMV positive MRC-5 cell line showing a well defined 2+ CPE was able to supplement the stealth viral growth enhancing activity of
15 medium X Vivo-15 used alone and was designated VEM for "viral enhancing medium". Titration of this medium showed that 20%-30% was adequate to provide significant growth enhancement. X Vivo-15 medium containing 20% each of both CMV and HHV-6 supernatants was more effective than medium
20 containing 20% or 30% CMV supernatant.

In a fifth aspect, a method of detecting a stealth virus is provided by: (1) inoculating a permissive cell line with a sample of said virus in a culture; (2) adding viral enhancing medium to the culture; and (3) detecting
25 *in vitro* a CPE in the permissive cell line.

In preferred embodiments the viral enhancing medium contains 30% boiled, filtered products derived from the supernatant of cultures of cytomegalovirus and 70% medium X Vivo-15; and the cell line is maintained in a culture
30 medium, that is frequently replaced.

In a sixth aspect culturing a virus is provided by: (a) cocentrifuging a sample of said virus with a permissive cell line of indicator cells; (b) inoculating the cell mixture into culture vessels; (c) adding viral
35 enhancing medium to the culture; and (d) detecting *in vitro* a CPE in the permissive cell line.

The PCC step in combination with the use of VEM will also improve the detection of the CPE associated with cytomegalovirus (CMV) and human herpesvirus 6 (HHV-6). The growth of CMV and HHV-6, however, are less dependent
5 on these modifications than that of stealth viruses. Furthermore, frequent refeeding of the cultures is not nearly as important for these viruses as it is for stealth viruses.

In preferred embodiments the virus is a stealth
10 virus, cytomegalovirus, or human herpesvirus-6.

The summary of the invention described in detail above is not intended to limit in any way the scope of the present invention which is defined in the appended claims.

4. Detailed Description of the Invention

15 Preferred embodiments of the present invention are described in detail below. However, the following description of the preferred embodiments is not intended to limit in any way the scope of the present invention, which is defined in the appended claims.

20 The present invention provides several culturing conditions that induce a cytopathic effect in a sample containing a stealth virus. These culturing conditions include using approximately 5%-10% fetal calf serum, refeeding the culture medium every 24 to 72 hours using
25 viral enhancing medium and using preculture centrifugation. These conditions are important for inducing a cytopathic effect. For example, the failure to replace the culture medium every 24 to 72 hours often prevents detection of the cytopathic effect. Another
30 example is provided by the fact that not all serum free medium induce a cytopathic effect. Indeed, the use of a basal medium such as minimal essential medium with 2% fetal calf serum and weekly refeeding of the cultures as is commonly practiced in most clinical virology
35 laboratories will not yield a cytopathic effect with

[illegible]

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circumstances a pure material or sample is desired and it would therefore be useful to detect a stealth virus in the material or sample.

It would be useful to know if a food or beverage contained a stealth virus. One could simply avoid consuming the food or beverage in that case as a matter of prudence in order to reduce the risk of contracting a stealth virus associated disease. Similarly, it would be useful to know if a sample of blood contained a stealth virus. One could then simply avoid that blood in order to reduce the risk of contracting a stealth virus associated disease. Many other potential sources of infection are identified herein. Thus, detecting the virus may allow individuals to avoid the virus and any diseases associated with the virus. In other words, the invention is useful for prevention of disease transmission by identifying potential sources of infection.

The present invention is also useful in a variety of other ways readily apparent to those skilled in the art.

20 6. Examples

This invention will be more fully understood with reference to the examples which follow. The following examples are intended to illustrate the invention, but not to limit its scope which is defined in the claims appended hereto. The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in the art in making and using the same, but are not intended in any way to otherwise limit the scope of the disclosure or the protection granted by a letters patent hereon.

In the following examples, the detection of the cytopathic effect was recorded as follows. This minimal level of CPE has to be transferable to secondary cultures for the culture to be recorded as a true positive (+). A + (one plus) positive effect indicates the presence of

multiple ~~or~~ enlarged refractile cells forming small clumps with indistinct cell borders are clearly seen in the cultures. A +/- (plus/minus) or equivocal effect refers to a minimal change in the culture in which the rounded cells are either not enlarged or non-refractile (possibly dead). Less than 4% of cultures from CFS patients have been recorded as equivocal, either because the CPE has been restricted to only a small region of the culture, or because it has failed to recur on secondary passage. These cultures have been excluded from analysis.

A ++ (two plus) strong positive response is recorded when vacuoles are clearly identified within enlarged, rounded, refractile cells and/or when multiple foci of cell clumping and/or destruction are apparent which are ringed by cells described as having a positive effect. A +++ (three plus) very strong positive effect refers to extensive ++ cellular changes throughout much of the culture and/or the appearance of large refractile vacuolated, foamy syncytial cell formation. Typical CPE's are shown in Patent Cooperation Treaty publication number WO 92/20787.

A benefit of using multiple indicator cell lines, is that the CPE appearances in the different cell lines sometime complement each other. For example, cell syncytia are usually best observed in monkey kidney cells. The smaller rounded refractile cells are usually best observed in the human fibroblasts. In over 80% of positive cultures, the CPE is clearly observable in at least 2 of the 3 cell lines. As with any viral induced CPE, it is important to confirm transmission to fresh cultures. In recipient cultures, the stealth virus induced CPE generally develops more rapidly than in the primary culture with at least the same level of intensity.

Although the appearances of the CPE share some features in common with those associated with cytomegalovirus (CMV) and herpes simplex virus (HSV) infection,

stealth virus cultures can be readily distinguished from cultures harboring these viruses. The CPE from HSV is much more rapid, occurring in a matter of days. The destruction is greater with large masses of dead cells.

5 The CPE from CMV tends to initially develop in smaller, looser clusters and evolves more slowly than observed with stealth viruses. The intracytoplasmic vacuolization and syncytia formation are far less prominent with CMV than with stealth viruses. The stealth virus infected cells
10 give more the appearance of "foamy cells" than does CMV. Indeed, with some stealth viral cultures, there appears to be a marked accumulation of lipid-like material attaching itself to the wall of the culture tube. Human CMV does not infect monkey derived cells.

15 7. Example 1: Culture Of Stealth Viruses - 7% FCS and Frequent Refeeding

Culture tubes containing human fibroblast (MRC-5), primary human foreskin fibroblast (MRHF) and rhesus monkey kidney (RMK) cells are each inoculated with a cellular
20 mixture of the buffy coat granulocytes and ficoll-hypaque separated lymphocytes derived from approximately the equivalent of one milliliter (ml) of heparinized blood. Typically, 5 ml of blood collected into a "green top" heparinized tube, are layered onto 3 mls of a ficoll-
25 hypaque lymphocyte separation medium. Following 20 minutes centrifugation at 1,500 rpm, the lymphocyte, which collect at the plasma: ficoll hypaque interface, and the "buffy coat", present on the top of the erythrocyte layer, are collected into approximately 1 ml. An effort is made
30 to minimize the number of erythrocytes in collecting all of the visible buffy coat.

The cell mixture is washed once in 1 ml of 199 medium containing 7% FCS and resuspended into 1 ml. Aliquots of 0.2 ml of the cells are added to culture tubes containing
35 1 ml of 199 medium plus FCS. The tubes are placed in an

incubator at 37°C for 45-60 minutes. The are then rinsed to remove macroscopically visible erythrocytes and other non-adherent cells. Rinsing (washing) consists of emptying the fluid content of the culture tube by decanting or by aspiration; adding 2 mls of medium or phosphate buffer saline; rocking the tube for several seconds to suspend erythrocytes; decanting the tube again. This important step is performed 2-10 times or until there are no macroscopically visible erythrocytes.

Two mls of medium with 7% FCS, are added and the tubes returned to a 37°C incubator. Cultures are maintained in the incubator at 37°C, with refeeding (replacement of old medium with medium) at 24, 48 and 72 hours. The tubes are examined microscopically after the 24 hour refeeding and, if residual erythrocytes are present, the tubes are rinsed in a manner similar to that performed at the 45-60 min. step. The tubes are refed three times each week by replacing the old medium with 2 mls of fresh medium. This procedure is designed to reduce the accumulation of a toxic component in the culture medium which tends to suppress viral growth. The tubes are examined three times per week for evidence of CPE (CPE). CPE is generally recognizable between 2-3 weeks after culturing.

A lack of regular refeeding of the cultures can result in a tendency for the CPE to abort and not to progress. This effect is not seen with CMV infected cultures. In unfed stealth virus cultures and even with regular refeeding, one can observe a toxic effect on many of the remaining cells. Culture cells appear to lose a degree of vitality and become duller in appearance compared to control cultures. The fibroblasts can assume somewhat of a pavement appearance, instead of the elongated shape. Some of these changes can be mimicked using 5 nM of the polyether marine toxin okadaic acid (Cohen et al., "Okadaic acid: A new probe for the study

of cellular regulation" *Trends Biochem. Sci.*, 15:98-102 (1990). CMV positive cultures do not demonstrate the toxic activity such as that observed with stealth viruses.

Moreover, the detection of CPE from CMV is readily
5 seen in cultures containing only minimal essential medium
and 2% FCS even without regular refeeding of cultures.
This is the routine medium used in most virology
laboratories and can be contrasted with the more enriched
medium 199 and 7% FCS that is used to culture stealth
10 viruses. The more enriched medium and the higher
concentration of FCS, help to neutralize the toxic,
stealth virus growth inhibiting effects, which would
otherwise occur in the cultures.

Although presumptive of stealth virus infection, the
15 CPE appearance may require additional confirmation for a
definitive conclusion of stealth virus infection.

8. Example 2: Viral Enhancing Medium (VEM)

In preparing viral enhancing medium a known positive CMV culture was passaged into a flask of MRC-5 cells and fed with X Vivo-15 medium. The cultures were observed for the development of CPE. The culture medium was changed at 1 week when approximately 50% of the cells showed signs of CPE, but before there was marked cellular destruction. This newly added medium was collected 48 hours later. It was centrifuged at 800 g for 20 minutes to remove cellular debris.

The medium was transferred to new tubes which were placed in a beaker containing water. The water was heated to boiling for 20 minutes. After cooling, the medium was filtered through a 0.45 micron Millipore filter. This material was diluted 30:70 into regular X Vivo-15 medium to constitute a "lot" of CMV derived VEM. Each lot of VEM is tested to confirm: i) that it does not contain any residual live CMV by adding the medium to MRC-5 cells; and ii) that it can promote the development of the CPE induced

by the prototype stealth virus by comparing the growth of the stealth virus in RMK cells containing VEM with that of similarly inoculated cells containing X Vivo-15 medium without supplement.

5 VEM has been tested on ten additional stealth viral isolates and clearly enhanced the growth of all of them. Two of these isolates are known to share CMV related sequences with the prototype stealth virus. Another isolate (from patient G.P.) shares antigens with HHV-6,
10 rather than CMV, and is considered an HHV-6 related stealth virus. Another isolate appears to have an adenoviral sequence. The use of VEM also reduced the time for a discernible CPE using fresh blood from two newly cultured CFS patients. It enhances the intensity of the
15 CPE and reduces the tendency for weekly positive cultures to revert to near normal appearance. VEM has also worked well in the cultures from the tissues of cats inoculated with the prototype stealth virus from patient D.W. allowing for clearly positive culture results. VEM
20 obtained from HHV-6 (strain GS) infected fibroblasts was similarly tested for its ability to promote the growth of CMV and HHV-6 associated stealth viruses. It worked well with both viral types with a discernable advantage on the HHV-6 related stealth virus from patient G.P., compared to
25 the CMV related stealth virus from patient D.W.

As a specific example, cultivation of a prototype stealth virus isolated from a CFS patient (initials D.W.) can be greatly enhanced by the addition to the culture of VEM comprising a 30% concentration of boiled, filtered
30 supernatants from cytomegalovirus (CMV) infected cultures. This addition helps remedy a deficiency of viral growth enhancing components coded for by the immediate-early (I-E) and probably other CMV related genes which are not detectable in the stealth virus from this patient.

9. Example 3: Viral Enhancing Medium and Pre-Culture Centrifugation

Human fibroblast (MRC-5), rhesus monkey kidney (RMK) and rabbit kidney (RK) cell lines were obtained from BioWhittaker, Inc., Walkersville, Maryland. The tubes were placed in a 37° incubator. The next day, the Delbecco's modified Eagles medium containing 2% FCS is replaced by medium 199 plus 7% FCS. The tubes were used to provide indicator cells for stealth viral cultures within the next 7 days. To establish the viral cultures, the contents of a single test tube of each of the indicator cell lines to be used were scraped from the tubes and washed once in X Vivo-15 medium.

The cells were gently resuspended into approximately 0.5 ml of medium and transferred to 2 ml Eppendorf tubes. Prior to this step, ficoll-hypaque separated lymphocytes from either heparinized or citrate treated whole blood, were obtained by layering 5 mls of anti-coagulated blood onto 3 mls of ficoll-hypaque solution in 12 ml conical tubes. The tubes were centrifuged for 20 min at 800 g. The banded lymphocytes were aspirated and transferred to a fresh tube for washing in 10 mls of medium. The lymphocytes were resuspended in approximately 1 ml. Aliquots of 0.2 ml of the lymphocytes were added to each of the Eppendorf tubes containing the harvested fibroblast indicator cells with a final aliquot stored for future studies.

The lymphocyte-fibroblast cell mixture was centrifuged at high speed for 3 minutes. The tightly-packed cell pellet was gently resuspended and transferred back to the tube from which the fibroblasts were originally obtained. Two mls of VEM (X Vivo-15 medium supplemented with 30% CMV supernatant) were added and the tubes are placed in an incubator at 37°C. The tubes were refed with VEM at 48 and 72 hours and thereafter 3 times per week.

Control cultures in which either lymphocytes from other individuals are used, or the fibroblasts were processed but with the exception of no added lymphocytes, were fed in parallel with the test cultures. Note, in 5 this revised protocol, buffy coat granulocytes are no longer routinely used since the contaminating erythrocytes tended to clump about the fibroblasts during the centrifugation step and were difficult to remove in subsequent washing of the cultures. Granulocytes may be 10 an important source of virus in some patients. If this proves to be so, leucocyte rich plasma will be obtained by dextran precipitation of the erythrocytes from anti-coagulated blood, or as an alternative, modifications of the ficoll-hypaque separation method can be used which 15 will separate both lymphocytes and granulocytes away from the erythrocytes. For example by using PMN isolation medium from Robbins Scientific Corp., Sunnyvale Ca. CSF and tissue extracts can be used in place of the lymphocytes.

20 The cultures were observed for a CPE which characteristically consists of rounding and swelling of the cells, formation of cell clumps which tend to disperse, appearance of intracellular granules/vacuoles and an overall foamy cell appearance often with prominent 25 accumulation of lipid-like material.

Table 3

Examples of the Enhanced Recovery and More Intense Development of CPE by a Stealth Virus from a CFS Patient Using Pre-Culture Centrifugation (PCC) and Viral Enhancing Medium (VEM).*

Method of Culturing	Time to CPE**	Intensity of CPE
Patient 1 Medium 199 + 7% FCS PCC and VEM	45 days 12 days	1-2+ 3+
Patient 2 Medium 199 + 7% FCS PCC and VEM	28 days 16 days	1-2+ 3+

* Medium X Vivo-15 containing 30% supplement of boiled filtered supernatant from a CMV culture also grown in medium X Vivo-15.

** Results are in RMK cells. Enhanced growth was also seen in MRC-5 cells.

10. Growth of Stealth Virus in Insect Cell Line

The Spodoptera fruiperda derived ovarian cell line Sf9 that is used routinely for the propagation of recombinant insect baculovirus was obtained from PharMigen, San Diego. It was maintained at 27°C in Grace's medium with 10% fetal calf serum. The stealth viruses from patients D.W., G.P., K.E. and B.B. were passaged into the insect cell line using 0.1 ml of cell-free supernatant from an infected MRC-5 human fibroblast culture. CPE was clearly seen within two days and progressed over the next several days.

The infected cultures showed enlarged foamy cell syncytia. Virus infectious for MRC-5 and for insect cell cultures was recoverable from the insect cell cultures to a dilution of 10^{-3} ml. Electron microscopic examination of the insect cultures infected with the virus from patient D.W. showed abundant herpes-like viral particles. In control studies, neither cytomegalovirus, human herpes virus 6, varicella zoster virus or Epstein-Barr virus

100-443886-100

The stealth virus isolated from patient D.W. (virus-X
5 infected MRC-5 cells) was deposited with the American Type
Culture Collection (ATCC) - 12301 Parklawn Drive,
Rockville, Maryland 20852, under the provisions of the
Budapest Treaty on the International Recognition of the
Deposit of Microorganisms for the Purposes of Patent
10 Procedures on 9-17-91, and were assigned accession no. VR-
2343.

The present invention is not to be limited in scope by the microorganisms deposited or the specific embodiments described herein since such embodiments are intended as but single illustrations of one aspect of the invention and any microorganisms which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference herein in their
25 entirety.

WHAT IS CLAIMED IS:

- 1 1. A method of detecting a stealth virus comprising
2 culturing a sample under conditions in which any stealth
3 virus in said sample is able to induce a cytopathic
4 effect.

- 1 2. The method of claim 1, in which the culturing is
2 performed by:
3 (a) inoculating a permissive cell line with a
4 sample; and
5 (b) detecting *in vitro* a cytopathic effect in
6 the permissive cell line.

- 1 3. The method of claim 2, in which the cell line is
2 maintained in culture medium, and further comprising
3 replacing the culture medium every 24 to 72 hours.

- 1 4. The method of claim 1, in which the sample is
2 taken from a patient diagnosed from physical symptoms as
3 having chronic fatigue syndrome.

- 1 5. The method of claim 2, wherein the cell line is
2 an insect cell line.

- 1 6. The method of claim 2, wherein the cell line is
2 Spodoptera fruiperda derived ovarian cell line Sf9.

- 1 7. A method for culturing a stealth virus
2 comprising:
3 (a) inoculating a permissive cell line with a
4 sample;
5 (b) neutralizing stealth virus associated toxic
6 activity; and
7 (c) detecting *in vitro* a cytopathic effect in the
8 permissive cell line.

1 8. The method of claim 7, in which the cell line is
2 maintained in culture medium and the neutralizing
3 comprises replacing the culture medium every 24 to 72
4 hours.

1 9. The method of claim 8, in which the cell line is
2 maintained in culture medium and the neutralizing
3 comprises adding a neutralizing agent to the culture
4 medium.

1 10. The method of claim 9, in which the neutralizing
2 agent is selected from the group consisting of 5% to 10%
3 fetal calf serum in medium 199, an antibody specific for,
4 the toxin and a chemical agent capable of reversing the
5 toxic activity.

1 11. The method of claim 7, wherein said sample is
2 derived from a human or animal source.

1 12. The method of claim 7, wherein said sample is
2 derived from food, an environmental substance, or an
3 object suspected as being a possible source of stealth
4 viral infection or transmission.

1 13. A method of culturing a stealth virus
2 comprising:

3 (a) inoculating a permissive cell line with a sample
4 of said virus in a culture;

5 (b) adding serum free medium to the culture; and

6 (c) detecting *in vitro* the presence of a cytopathic
7 effect in the permissive cell line.

1 14. The method of claim 12, wherein the serum-free
2 medium is medium X Vivo-15.

1 15. A method for culturing a stealth virus
2 comprising:

3 (a) co-centrifuging a sample of said virus with a
4 permissive cell line of indicator cells;

5 (b) inoculating the cell mixture into culture
6 vessels; and

7 (c) detecting *in vitro* a cytopathic effect in the
8 permissive cell line.

16. The method of claim 14, further comprising
2 adding cytomegalovirus supernatant to the culture.

17. The method of claim 14, further comprising
2 adding viral enhancing medium to the culture.

18. The method of claim 14, further comprising
2 refeeding the culture medium every 24 to 72 hours.

1 19. A method for culturing a stealth virus
2 comprising:

3 (a) inoculating a permissive cell line with a sample
4 of said virus in a culture;

5 (b) adding viral enhancing medium to the culture;
6 and

7 (c) detecting *in vitro* a cytopathic effect in the
8 permissive cell line.

20. The method of claim 18, wherein said viral
2 enhancing medium contains 30% boiled, filtered products
3 derived from the supernatant of cultures of
4 cytomegalovirus and 70% medium X Vivo-15.

21. The method of claim 18, in which the cell line
is maintained in culture medium, and further comprising
3 replacing the culture medium every 24 to 72 hours.

- 1 22. A method for culturing a virus comprising:
2 (a) cocentrifuging a sample of said virus with a
3 permissive cell line of indicator cells;
4 (b) inoculating the cell mixture into culture
5 vessels;
6 (c) adding viral enhancing medium to the culture;
7 and
8 (d) detecting *in vitro* a cytopathic effect in the
9 permissive cell line.

23. The method of claim 21, wherein said virus is a
2 stealth virus.

24. The method of claim 21, wherein said virus is
2 cytomegalovirus.

25. The method of claim 23, wherein said virus is
2 human herpesvirus-6.

0903071-050909

Abstract

A method of detecting a stealth virus is provided by culturing a sample under conditions in which any stealth virus in the sample is able to induce a cytopathic effect.

- 5 A method for culturing a virus is also provided by (a) cocentrifuging a sample of said virus with a permissive cell line of indicator cells; (b) inoculating the cell mixture into culture vessels; (c) adding viral enhancing medium to the culture; and (d) detecting in vitro a
10 cytopathic effect in the permissive cell line.

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COMBINED DECLARATION AND POWER OF ATTORNEY
(Continuation or CIP Application)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the

invention entitled STEALTH VIRUS DETECTION IN THE CHRONIC FATIGUE SYNDROME

_____ the specification of which
_____ X is attached hereto.

_____ was filed on _____ as Application

Serial No. _____ and was amended

on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56 (a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):

_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	_____ Yes	_____ No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	_____ Yes	_____ No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	_____ Yes	_____ No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>07/887,502</u> (Application Serial No.)	<u>May 22, 1992</u> (Filing Date)	<u>Pending</u> (Status) (patented, pending, abandoned)
<u>07/704,814</u> (Application Serial No.)	<u>May 23, 1991</u> (Filing Date)	<u>Abandoned</u> (Status) (patented, pending, abandoned)
<u>07/763,039</u>	<u>September 20, 1991</u>	<u>Abandoned</u>

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Richard J. Warburg, Reg. No. 32,327

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Address all telephone calls to Richard J. Warburg, Esq. at telephone no. (619) 552-8400.

Address all correspondence to Richard J. Warburg, Esq., LYON & LYON, 611 West Sixth St., 34th Floor, Los Angeles, CA 90017

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full name of sole or first inventor William John Martin

Inventor's signature W. John Martin Date 11/9/93

Residence Pasadena, California

Citizenship Australian

Post Office Address 1634 Spruce Street, Pasadena, CA 91030

Full name of second inventor _____

Inventor's signature _____ Date: _____

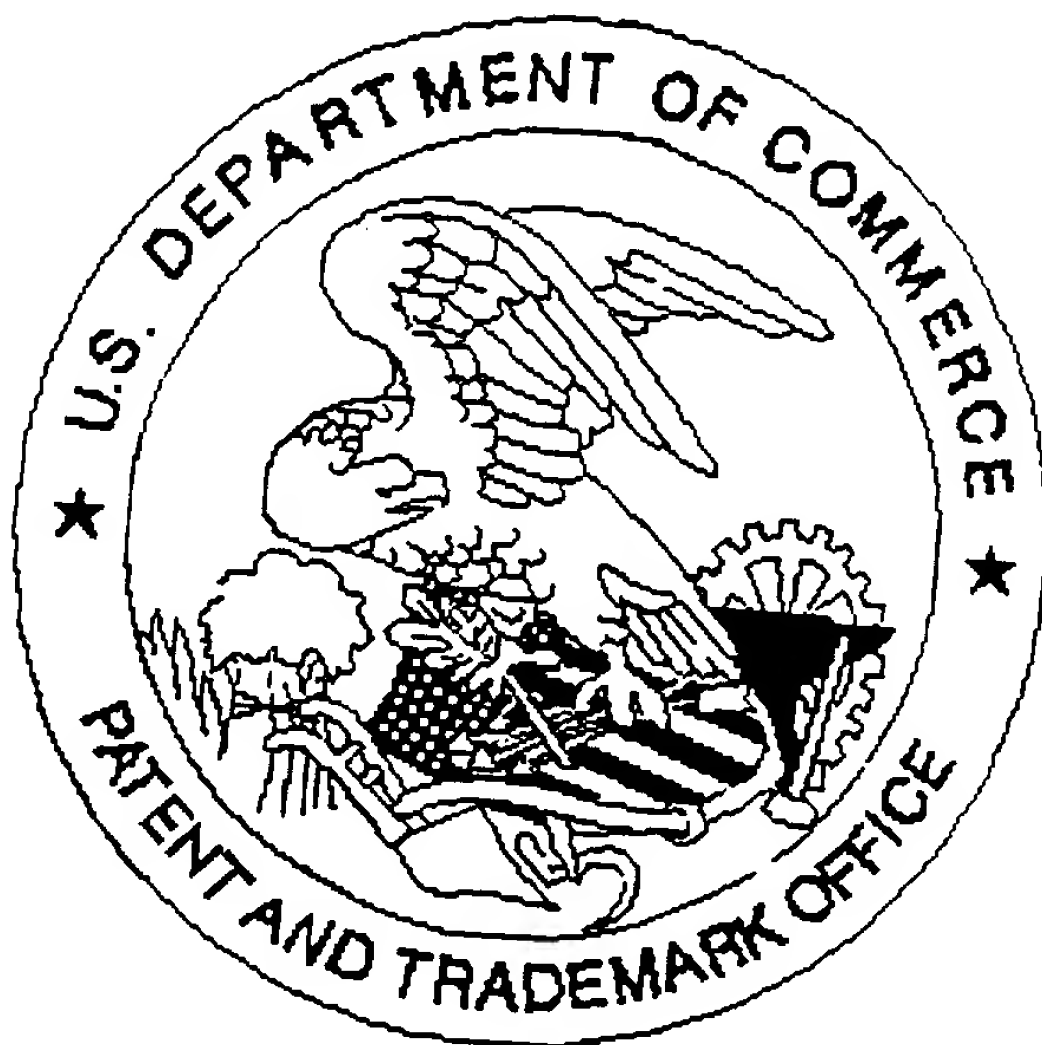
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